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Effect of collagenase concentration on the isolation of small adipocytes from human buccal fat pad

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Abstract: Dedifferentiated fat (DFAT) cells were isolated from mature adipocytes using the ceiling culture method. Recently, we successfully isolated DFAT cells from adipocytes with a relatively small size (<40 μm). DFAT cells have a higher osteogenic potential than that of medium adipocytes. Therefore, the objective of this study was to determine the optimal concentration of collagenase solution for isolating small adipocytes from human buccal fat pads (BFPs). Four concentrations of collagenase solution (0.01%, 0.02%, 0.1%, and 0.5%) were used, and their effectiveness was assessed by the number of small adipocytes and DFAT cells isolated. The total number of floating adipocytes that dissociated with 0.02% collagenase was 2.5 times of that dissociated with 0.1% collagenase. The number of floating adipocytes with a diameter of ≤29 μm that dissociated with 0.02% collagenase was thrice of those dissociated with 0.1% and 0.5% collagenase. The number of DFAT cells that dissociated with 0.02% collagenase was 1.5 times of that dissociated with 0.1% collagenase. In addition, DFAT cells that dissociated with 0.02% collagenase had a higher osteogenic differentiation potential than those that dissociated with 0.1% collagenase. These results suggest that 0.02% is the optimal collagenase concentration for isolating small adipocytes from BFPs.

Keywords: adipocytes; buccal fat pads; collagenase; dedifferentiated fat (DFAT) cells; isolation.

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Introduction

Mature adipocytes possessing a single, large lipid droplet are the most abundant cells in the adipose tissue. These cells are generally considered to be in the terminal stage of differentiation, having lost their proliferative ability. We previously established a preadipocyte-like cell line called dedifferentiated fat (DFAT) cells through the asymmetrical division of mature adipocytes from subcutaneous fat tissue using the ceiling culture method, which relies on the inherent buoyancy of adipocytes in the absence of any specific factors (1-3). Unlike terminally

differentiated adipocytes, DFAT cells showed strong and stable proliferation ability. In addition, they differentiated *in vitro* and *in vivo* into not only adipocytes but also osteoblasts, chondrocytes, skeletal myocytes, smooth muscle cells, cardiomyocytes, vascular endothelial cells, and neural cells under appropriate culture conditions (4-10).

Adipose-derived stem cells (ASCs), also known as MSCs, have been widely studied as potential stem cells in regenerative medicine (11-13). Both ASCs and DFAT cells can be isolated from subcutaneous adipose tissue (6) using the same method in which adipose tissues are enzymatically digested with collagenase and then centrifuged to separate the stromal vascular fraction (SVF) from adipocytes; adipocytes float because of their lipid droplets, whereas SVF is sedimented. ASCs can be isolated from SVF using ASC surface markers by flow cytometry (11-13).

Oral and maxillofacial research studies demonstrated that DFAT cells and ASCs could potentially contribute to the generation of skeletal bone and periodontal tissue in animals (14,15). However, our data suggested that the osteoblastic differentiation ability of rat DFAT cells is higher than that of ASCs (16,17), which was consistent with the findings of other researchers (18). Taken together, these data indicate that DFAT cells are a particularly promising resource for bone and periodontal tissue regeneration, although their clinical application has not been evaluated.

The oral cavity contains a mass of specialized fatty tissue called the buccal fat pad (BFP or Bichats fat pad), which is distinct from subcutaneous fat (19). BFP is a simple lobulated mass consisting of a central body and four extensions (buccal, pterygoid, pterygopalatine, and temporal), and is located between the masseter and buccinators muscles and ascending mandibular ramus and zygomatic bone (19,20). Harvesting tissue from BFP is not a complicated procedure; it requires a minor incision and local anesthesia and causes minimal donor-site trauma when performed by a dentist or oral surgeon. Recent reports have shown that ASCs from BFP can differentiate into chondrocytes, osteoblasts, and adipocytes *in vitro*. In addition, BFP-derived ASCs successfully generated bone tissue in the back subcutaneous pockets of nude mice (21), and DFAT cells were generated from mature adipocytes harvested from BFPs (18,22). Taken together, these data indicate that BFP might be a potential source of DFAT cells for bone and periodontal tissue engineering.

Recently, we isolated mature adipocytes from BFPs and examined their size distribution (23). The average diameter of adipocytes ranges between 60 and 80 μm; however, we observed that the number of cells with diameter in the abovementioned range was only one-third of that of the largest cells which had a diameter of <40 μm. Thus, we succeeded in generating DFAT cells from small adipocytes (<40 μm). DFAT cells obtained from small adipocytes have several advantages compared with those obtained from large adipocytes (average size, 60-80 μm). First, the efficiency of dedifferentiation into DFAT cells was higher for small adipocytes than for large adipocytes, sugegsting that a large number of DFAT cells can be obtained from the same number of small adipocytes. In addition, DFAT cells generated from small adipocytes showed higher osteogenic potential than that of DFAT cells generated from large adipocytes. Taken together, we concluded that DFAT cells obtained from small adipocytes have an advantage over those obtained from large adipocytes in bone and periodontal tissue engineering.

When mature adipocytes are isolated from BFPs, enzymatic digestion is necessary to facilitate the efficient breakdown of extracellular structures in adipose tissue. Generally, adipose tissue is treated with collagenase to digest the native collagen in the triple helix region. In the first study that reported the ceiling culture method, a 0.2% collagenase solution was used to isolate mature adipocytes (24). However, other researchers have shown that 0.1% (6), 0.3% (10), 0.2% (25), and 0.075% (26) collagenase can also be used. To the best of our knowledge, there are no studies defining the optimal concentration of collagenase for isolating small adipocytes from BFPs. Here we examined the appropriate concentration of collagenase for isolating a large number of DFAT cells from small adipocytes obtained from human BFP. We examined four concentrations of collagenase: 0.1, 0.2, 1.0, 5.0 mg/mL. For each concentration, we determined the number of small adipocytes and DFAT cells and characteristics of DFAT cells.

Materials and Methods

Isolation of mature adipocytes and culture of DFAT cells

Human BFP samples were obtained from 10 patients (cases 1-10) who underwent oral and maxillofacial surgery at the Nihon University School of Dentistry Dental Hospital (Table 1). All subjects were healthy and had no systemic conditions. The patients gave a written informed consent, and the Ethics Committee of Nihon University School of Dentistry approved the study (EP2008-8). DFAT cells were isolated using the ceiling culture method (24,27). Briefly, approximately 5-10 g of BFP was harvested from each patient, minced

Table 1 Buccal fat pad obtained from 10 patients

Case	Age (years, months)	Sex	Surgery	Amount (g)
1	26, 10	F	Le fort I, SSRO	4.5
2	19, 2	F	Le fort I, SSRO	9.9
3	24, 4	F	Le fort I, SSRO	8.5
4	19, 4	M	Le fort I, SSRO	9.6
5	18, 10	F	Le fort I, SSRO	9.8
6	36, 0	F	Le fort I, SSRO	5.2
7	45, 4	M	Le fort I, SSRO	4.8
8	21, 2	F	Le fort I, SSRO	10.0
9	36, 1	F	Le fort I, SSRO	8.5
10	20, 4	M	Le fort I, SSRO	7.0

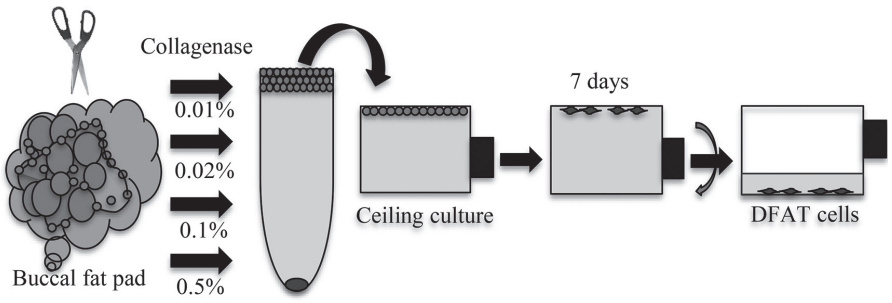

Fig. 1 Method used for isolating dedifferentiated fat (DFAT) cells from buccal fat pad (BFP) tissue. BFP tissue was minced and dissociated with 0.01%, 0.02%, 0.1%, or 0.5% collagenase solution, and cell suspensions were filtered and centrifuged. Floating adipocytes were harvested and cultured in culture flasks completely filled with Dulbecco's modified Eagle's medium containing 20% fetal bovine serum for 7 days. The flasks were inverted and DFAT cells were cultured using conventional methods.

into small pieces, and then dissociated using collagenase solution. Four concentrations of collagenase solution (0.01%, 0.02%, 0.1%, and 0.5%) corresponding to 0.1, 0.2, 1.0, and 5.0 mg/mL (C6885; Sigma-Aldrich, St. Louis, MO, USA), respectively, in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 2.0% bovine serum albumin (Sigma-Aldrich) were used at 37°C (pH 7.4) for 1 h with gentle agitation. The cell suspensions were filtered through a 100-μm nylon mesh and centrifuged at 700 rpm for 1 min. Isolated floating adipocytes were placed in 25-cm^2 culture flasks (BD Falcon, San Jose, CA, USA) that were completely filled with DMEM supplemented with 20% fetal bovine serum (FBS; 13B103, Sigma-Aldrich) and 1% antibiotic solution as a growth medium (GM). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The flasks were inverted for floating adipocytes to adhere to the upper surface of the flask. This method is known as the ceiling culture method (Fig. 1). After 7 days, the flasks were inverted again, and the medium was replaced with fresh GM. The medium was subsequently replaced twice a week. At 80% confluence, the cells were passaged and used for further experiments.

Determination of cell size and number of floating adipocytes

To determine the cell size distribution, the floating adipocytes were suspended in 1 mL DMEM, and cell number and diameter were measured using Coulter Counter Multisizer 3 (Beckman Coulter, Miami, FL, USA). Cell diameters were categorized as follows: <29, 30-39, 40-49, 50-59, 60-69, 70-79, 80-89, and 90-99 μm. Measurements were performed thrice. Results are presented as mean ± standard deviation.

Nile red fluorescence staining of floating adipocytes

To determine whether cells contained lipid-filled vacuoles, floating cells dissociated with 0.02% and 0.1% collagenase solutions were collected and incubated for 20 min with Nile red fluorescence dye (AdipoRed assay, Lonza, Walkerslive, MD, USA) and 5 μg/mL Hoechst 33342 (Sigma-Aldrich) to visualize lipid droplets and nuclei, respectively. Images of staining were captured using immunofluorescence microscopy (Keyence, Osaka, Japan).

Determination of DFAT cell number

After digestion, floating cells in the top layer of 50 mL

Table 2 Sequence of primer pairs for reverse transcription-polymerase chain reaction (RT-PCR)

Primers	Primer sequence	Size (bp)
c-MYC	F 5'-GCG TCC TGG GAA GGG AGA TCC GGA GC-3'	328
	R 5'-TTG AGG GGC ATC GTC GCG GGA GGC TG-3'	
KLF4	F 5'-ACG ATC GTG GCC CCG GAA AAG GAC C-3'	397
	R 5'-TGA TTG TAG TGC TTT CTG GCT GGG CTC C-3'	
OCT3/4	F 5'-GAC AGG GGG AGG GGA GGA GCT AGG-3'	144
	R 5'-CTT CCC TCC AAC CAG TTG CCC CAA AC-3'	
SOX2	F 5'-GGG AAA TGG GAG GGG TGC AAA AGA GG-3'	151
	R 5'-TTG CGT GAG TGT GGA TGG GAT TGG TG-3'	
PPARγ2	F 5'-TCA GGT TTG GGC GGA TGC-3'	147
	R 5'-TCA GCG GGA AGG ACT TTA TGT ATG-3'	
RUNX2	F 5'-CCC TGA ACT CTG CAC CAA GT-3'	128
	R 5'-CGT CAT CTG GCT CAG GTA GG-3'	
SOX9	F 5'-AGC GAA CGC ACA TCA AGA C-3'	85
	R 5'-CTG TAG GCG ATC TGT TGG GG-3'	
GAPDH	F 5'-GCA CCG TCA AGG CTG AGA AC-3'	138
	R 5'-TGG TGA AGA CGC CAG TGG A-3'	

tubes were placed in 12.5 cm^2 flasks (1.0×10^4 cells/flask) for 7 days. The flasks were inverted so that DFAT cells were located at the bottom (floor) of the flask. The cells were passaged after 3 days and then counted (passage 1) using a Countess automated cell counter (Invitrogen, Eugene, OR, USA). Counts were recorded thrice. Results are presented as the mean ± standard deviation.

Analysis of cell surface markers

The immunophenotype of DFAT cells at passage 2 was identified using flow cytometry as previously described (23). The cells grown to 60% confluence were suspended at a density of 5×10^5 cells per tube and labeled using antibodies for the following antigens: CD13 (PE-CD13, aminopeptidase N), CD44 (FITC-CD44, hyaluronate recptor, and phagocytic glycoprotein-1), CD45 (FITC-CD45, leucocyte common antigen), CD73 (PE-CD73, NT5E), CD90 (APC-CD90, Thy-1), CD105 (APC-CD105, endoglin), CD271 (PE-CD271, NGFR), and STRO-1 (FITC-STRO1) (all from BD Biosciences, San Jose, CA, USA). Also, control cells were incubated with isotype-matched mouse anti-human IgGs and used as negative controls. Dead cells were identified by staining with 1 μg/mL propidium iodide (Sigma-Aldrich). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRI reagent (Cosmo Bio, Tokyo, Japan) according to the manufacturer's protocol when the cells had reached 60% confluence in 100-mm culture dishes. RNA concentration was determined using NanoDrop 1000 (Thermo Fisher Scientific Inc., Yokohama, Japan), and cDNA generated from 1 μg of total RNA was amplified using the ReverTra Ace qPCR-RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. PCR amplification was performed using a PCR Thermal Cycler Dice (Takara Bio Inc., Ohtsu, Japan): denaturation at 98°C for 10 s, annealing at 58°C (for GAPDH, KLF-4, OCT3/4, SOX2, RUNX2, PPARγ2, and SOX9) or 62°C (for c-MYC) for 30 s, and extension at 72°C for 60 s with 35 amplification cycles. The primer sequences are shown in Table 2.

Colony forming unit-fibroblast (CFU-F) assay

Cells at passage 2 were seeded onto 6-well plates (1,000 cells per well) and incubated in GM for 10 days. The cells were then stained with 0.05% toluidine blue (Sigma-Aldrich). Aggregates with >50 cells were scored as a colony. Each test was performed thrice. Results are presented as mean ± standard deviation.

Cell proliferation assay

Cells at passage 2 were seeded onto 6-well plates (1,000 cells per well) and counted using a Cell Counting Kit-8 (WST-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions on the indicated days as previously described (28). Each test was performed thrice. Results are presented as mean ± standard deviation.

Cell cycle analysis

Cell cycle analysis was performed using Click-iT EdU Flow Cytometry Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions as previously described (28,29). Briefly, cells at passage 2 were cultured onto 100-mm culture dishes (1.0×10^5

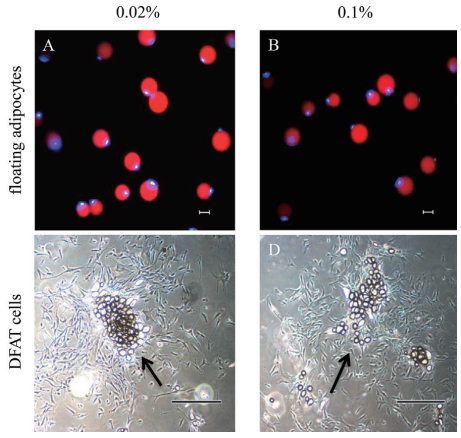

Fig. 2 Isolated mature adipocytes and cultured dedifferentiated fat (DFAT) cells obtained using 0.02% and 0.1% collagenase. (A, B) Microscopic analysis of floating adipocytes stained with Nile red and Hoechst 33342. Scale bars represent 40 μm. (C, D) Morphology of DFAT cells on day 7 of culture. When the flasks were inverted, DFAT cells formed colonies at the bottom of the flasks (arrows). Scale bars represent 500 μm.

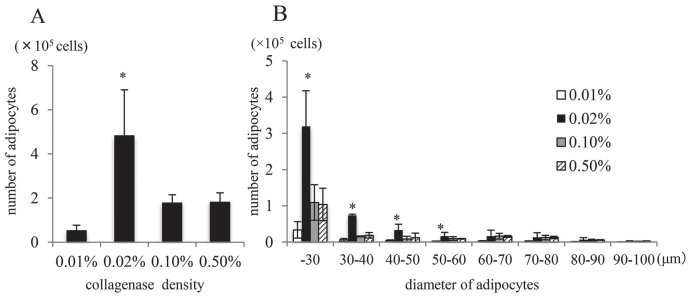

Fig. 3 Number of floating adipocytes dissociated with 0.01%, 0.02%, 0.1%, and 0.5% collagenase. (A) Total number of floating adipocytes dissociated with four different concentrations of collagenase solutions. (B) Number of floating adipocytes dissociated with four different concentrations of collagenase solutions based on cell diameter. Columns indicate mean values, and error bars indicate standard deviation ($n = 3$, $*P < 0.05$).

cells/dish) in GM for 3 days and treated with 10 mM of EdU. Cells were exposed to a click-reaction mixture containing blue azide (C10418), and the percentages of cells in the S, G2/M, and G1 phases were analyzed using the BD FACS Aria flow cytometer (BD Biosciences).

Osteogenic differentiation

Cells at passage 2 were seeded onto 12-well plates (1.0×10^4 cells per well) in GM. The medium was replaced with osteogenic induction medium consisting of GM supplemented with 100 nM dexamethasone, 10 mM β-glycerol disodium salt hydrate (Sigma-Aldrich), and 50 mM L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Pure Chemical Industries, Osaka, Japan) or with fresh GM (control). Cells were then cultured in OIM or GM for 21 days, and alkaline phosphatase activity was measured using a Pierce BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) on the indicated days according to the manufacturer's instructions. To detect calcium deposition, cells were stained with alizarin red S solution (pH 4.0, Wako). In addition, calcium accumulation was determined by measuring Ca^{2+} uptake into the cell culture at the indicated days using the Calcium E-Test kit (Wako) according to the manufacturer's instructions. Each test was performed thrice. Results are presented as mean ± standard deviation.

Adipogenic differentiation

Cells at passage 2 were seeded onto 12-well plates (1.0×10^4 cells per well) in GM for 7 days, and the medium was replaced with adipogenic induction medium consisting of GM supplemented with 1 mM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), and 1 × insulin-transferrin-selenium-A (Gibco, Grand Island, NY, USA) or GM (control). Cells were cultured 21 days and stained with Oil red O (Wako) as previously described (30,31). Positively stained cells were counted, and their total proportion was calculated.

Statistical analysis

All statistical analyses were performed using Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical differences were evaluated using the Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

Results

Isolation of mature adipocytes from BFPs

Nile red and Hoechst 33342 staining used for visualizing lipid droplets and nuclei, respectively, showed that the vast majority (≥95%) of floating adipocytes that dissociated in 0.02% and 0.1% collagenase solutions were monovacular adipocytes with a single nucleus (Fig. 2A, B).

Adipocyte number and size

The numbers of floating adipocytes and their sizes at each collagenase concentration are shown in Fig. 3. The total numbers of floating adipocytes that dissociated with 0.02%, 0.1%, and 0.5% collagenase were 4.8×10^5, 1.8

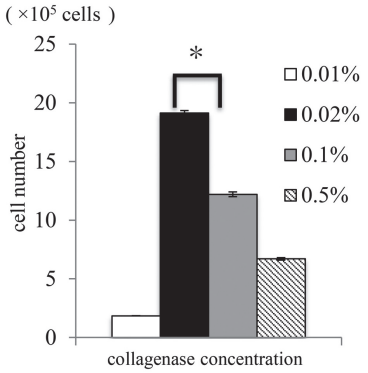

Fig. 4 Number of DFAT cells at passage 1 generated from adipocytes dissociated with 0.01%, 0.02%, 0.1%, and 0.5% collagenase. Columns indicate mean values, and error bars indicate standard deviation ($n = 8$, $*P < 0.05$).

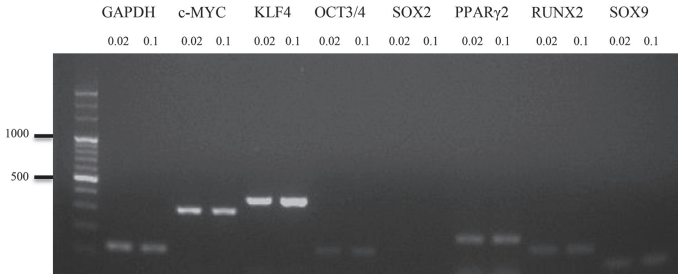

Fig. 5 Reverse transcription-polymerase chain reaction for DFAT cells obtained using 0.02% and 0.1% collagenase. Levels of mRNA encoding embryonic stem cell markers (c-MYC, KLF-4, OCT3/4, and SOX2), transcription factors expressed by osteogenic, adipogenic, and chondrogenic lineages (RUNX2, PPARγ2, and SOX9, respectively), and GAPDH for one donor are shown. A similar gene expression pattern was observed when the analysis was repeated using cell populations from three other donors.

Table 3 Expression of cell surface markers

% (w/v)	CD13	CD44	CD45	CD73	CD90	CD105	CD271	STRO-1
0.02	99.2	73.1	0.3	93.8	94.9	98.9	0.5	0.1
0.1	99.0	70.6	0.2	97.5	94.3	98.1	0.2	0.2

$\times 10^5$, and 1.8×10^5 cells, respectively; thus, the number of adipocytes that dissociated with 0.02% collagenase was 2.5 times than those dissocated with 0.1% and 0.5% collagenase. Moreover, for floating adipocytes with a diameter of <30 μm, the number of cells that dissociated with 0.02%, 0.1%, and 0.5% collagenase was 3.2×10^5, 1.1×10^5, and 1.1×10^5 cells, respectively. The number of cells that dissociated with 0.02% collagenase was thrice than those dissociated with 0.1% and 0.5% collagenase. Also, for floating adipocytes with diameters ranging between 30 and 90 μm, the number of cells that dissociated with 0.02%, 0.1%, and 0.5% collagenase was 7.2×10^4, 1.8×10^4, and 1.8×10^4 cells, respectively, and for cells with diameters ranging between 40 and 49 μm, the number of cells that dissociated with 0.02%, 0.1%, and 0.5% collagenase was 3.3×10^4, 1.5×10^4, and 1.6×10^4 cells, respectively; the number of cells that dissociated with 0.02% collagenase was twice and thrice of that dissociated with 0.1% and 0.5% collagenase, respectively. However, the number of floating adipocytes with diameters ranging between 60 and 69 μm was 1.1×10^4, 1.1×10^4, and 1.0×10^4 cells for 0.02%, 0.1%, and 0.5% collagenase, respectively; there were no significant differences between these cell numbers.

Number of DFAT cells

The adherent cells generated from adipocytes dissociated with 0.02% and 0.1% collagenase exhibited spindle-shaped morphology and were deemed to be DFAT cells (Fig. 2C, D). The number of DFAT cells at passage 1 that dissociated with 0.01%, 0.02%, 0.1%, and 0.5% collagenase was 1.8×10^5, 1.9×10^6, 1.2×10^6, and 6.7×10^5 cells, respectively; therefore, the number of DFAT cells that dissociated with 0.02% collagenase was approximately 1.6 times of that dissociated with 0.1% collagenase (Fig. 4).

Characterization of DFAT cells

The characteristics of DFAT cells generated from mature adipocytes dissociated with 0.02% and 0.1% collagenase were assessed. The surface markers expressed by these groups of DFAT cells are shown in Table 3. The common bone marrow stromal cell markers (CD13, CD73, CD90, and CD105) were expressed in more than 90% of cells. CD45, CD271, and STRO-1 expression levels were very low (<1%). There were no significant differences in the expression levels of cell surface markers between DFAT cells dissociated with 0.02% and 0.1% collagenase. On analyzing gene expression levels using RT-PCR, both the groups of DFAT cells expressed mRNA encoding c-MYC, KLF4, OCT3/4, RUNX2, PPARγ2, SOX9, and GAPDH, but not SOX2 (Fig. 5). This pattern was observed in cell populations from a total of four donors.

Cell cycle analysis showed that the proportion of DFAT cells in the S and G2/M phases was almost similar for cells dissociated with 0.02% and 0.1% collagenase (Table 4). This pattern was observed in cell populations from a total of four donors. DFAT cells dissociated with

Table 4 Flow cytometry analysis of cell cycle progression

Case	S (%)		G2/M (%)		G1 (%)	
	0.02%	0.1%	0.02%	0.1%	0.02%	0.1%
5	1.5	2.8	8.2	8	88.4	84.3
6	3.3	3.4	8.6	9.8	85.3	83.7
7	7.2	9	11.8	12.5	74.3	72.9
8	3.5	3	12.4	12.1	78.2	79.2

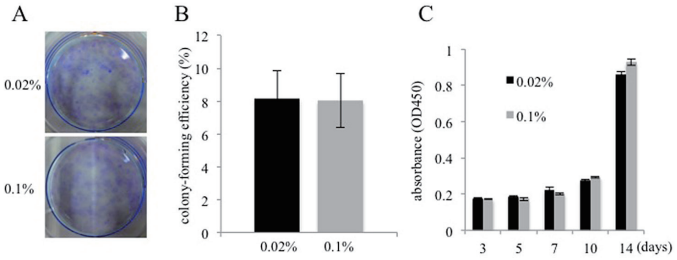

Fig. 6 Colony forming unit-fibroblasts (CFU-Fs) and cell proliferation potential after using 0.02% and 0.1% collagenase. (A) Colonies stained with toluidine blue after 10 days of culture. (B) CFU-Fs (aggregates with >50 cells) were counted in triplicate to determine the colony forming efficiency. Columns indicate mean values, and error bars indicate standard deviation (n = 5). (C) Results of WST-8 assay performed 3, 5, 7, 10, and 14 days after the cells were cultured in growth medium for 14 days. Columns indicate mean values, and error bars indicate standard deviation (n = 5).

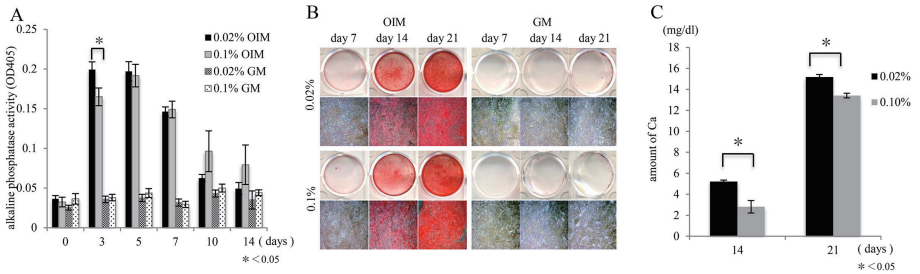

Fig. 7 Osteogenic differentiation potential of DFAT cells after using 0.02% and 0.1% collagenase. (A) Alkaline phosphatase activity measured on days 0, 3, 5, 7, 10, and 14. Alkaline phosphatase activity at day 3 in cells cultured with 0.02% collagenase was greater than that of cells cultured with 0.1% collagenase. Columns indicate mean values, and error bars indicate standard deviation. (B) Results of Alizarin red S staining for matrix mineralization and quantification performed on days 7, 14, and 21. Mineralized nodules were observed in OIM, but not GM. (C) Amount of calcium, a marker of terminal-stage osteoblastic differentiation, was higher in cells obtained with 0.02% collagenase than in cells obtained with 0.1% collagenase on days 14 and 21. Columns indicate mean values, and error bars indicate standard deviation (n = 3).

0.02% and 0.1% collagenase both formed colonies. Also, there were no significant differences in CFU-F and cell number between DFAT cells dissociated with 0.02% and 0.1% collagenase (Fig. 6A-C).

Osteogenic differentiation potential was assessed based on ALP activity, alizarin red S staining, and amount of calcium deposition (Fig. 7A-C). DFAT cells dissociated with 0.02% had higher ALP activity at day 3 than those dissociated with 0.1% collagenase, and only cells cultured in osteogenic induction medium were stained with alizarin red S, indicating matrix mineralization. The amount of calcium deposition in DFAT cells dissociated with 0.02% collagenase was significantly higher than that of DFAT cells dissociated with 0.1% collagenase on days 14 and 21 of osteogenic induction culture (Fig. 7C). Adipogenic differentiation potential was examined by Oil red O staining after culture in adipogenic induction medium (Fig. 8A) and counting the Oil red O-positive cells (Fig. 8B). A large number of Oil red O-positive cells was found in the adipogenic induction medium, but not in GM. However, for adipogenic differentiation potential, there were no significant differences between cells

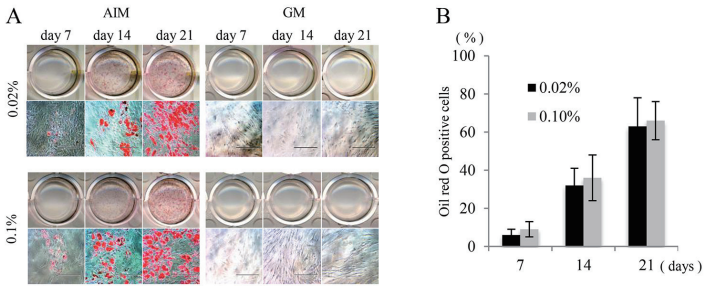

Fig. 8 Adipogenic differentiation potential of DFAT cells after using 0.02% and 0.1% collagenase. (A) Intracellular lipids visualized by Oil red O staining on days 7, 14, and 21, showing large numbers of Oil red O-positive cells in the adipogenic induction medium (AIM), but not in growth medium (GM). The patterns for 0.02% and 0.1% collagenase groups were similar. (B) Proportion of Oil red O-stained cells on days 7, 14, and 21. Columns indicate mean values, and error bars indicate standard deviation (n = 4).

dissociated with 0.02% and those with 0.1% collagenase.

Discussion

In this study, we examined the effectiveness of four collagenase concentrations based on the number of mature adipocytes obtained from BFPs and that of DFAT cells obtained from mature adipocytes. The isolation of mature adipocytes from adipose tissues and use of collagenase to break down the native collagen matrix that holds the connective tissue have been applied for several decades (4,6,32,33). Crude collagenase type II, which was used in our study, has been demonstrated to be suitable for digesting adipose tissue and releasing mature adipocytes with undamaged cell surface receptors for insulin (32,33). The optimal concentration of collagenase required to reliably obtain a large number of mature adipocytes from adipose tissue remains unknown.

Viable mature adipocytes were obtained with all four collagenase concentrations, although different concentrations produced significantly different numbers of mature adipocytes. The standard concentration for isolating mature adipocytes to obtain DFAT cells described by Matsumoto et al. (6) was 0.1%. The conventional method of isolating DFAT cells using 0.1% collagenase yielded significantly fewer cells than those yielded using 0.02% collagenase. We therefore investigated why the largest number of mature adipocytes was obtained with 0.02% collagenase. Recently, adipocyte size in the abdominal depot was identified to be a significant predictor for type 2 diabetes mellitus (34), and adipocyte size was found to be an important determinant of the secretion of several adipokines (35). Recently, small adipocytes have been shown to have greater proliferative ability *in vitro* than that of larger adipocytes (36). Owing to the normal size variation among adipocytes, we examined the cell size distribution based on the diameter of floating adipocytes obtained with different collagenase concentrations. First, we established that almost 100% of the floating cells were mature adipocytes using Nile red and Hoechst staining. Interestingly, when BFP tissue was exposed to 0.02% collagenase, cell numbers of adipocytes of <59-μm diameter considerably differed compared with 0.01%, 0.1%, and 0.5% collagenase. In particular, compared with tissues digested using other collagenase concentrations, significantly more adipocytes with a diameter of ≤30 μm were obtained using 0.02% collagenase. The number of cells obtained with 0.02% collagenase digestion wasthrice of that obtained with 0.1% collagenase digestion. In addition, more adipocytes ranging from 30 to 59 μm in diameter were obtained with 0.02% collagenase than with other concentrations. These results suggested that the difference in the total adipocyte numbers obtained from BFPs with different collagenase concentrations were largely due to differences in the numbers of small adipocytes.

The process of isolating adipocytes from adipose tissue requires a level of tissue disruption that facilitates effective cell release without damage or destruction. It is well known that high collagenase concentrations and prolonged digestion times can result in adipocyte damage (37). Moreover, Markarians et al. investigated different concentrations of trypsin for isolating ASCs obtained from adipose tissues and found that a lower concentration was better than a higher concentration (38). Taken together, these findings indicated that enzyme concentration is an important factor in the isolation of adipocytes from adipose tissue. Collagenase concentration must be low enough to minimize cell damage (e.g., 0.01%), but not so low as to destroy the native collagen matrix. Further research on methods for isolating mature adipocytes from adipose tissue is needed to determine the contribution of other factors in the protocol outcomes.

From a practical standpoint, the best protocol is the one that results in the highest number of DFAT cells from mature adipocytes for cell transplantation. On counting the number of DFAT cells after one passage, 0.02% collagenase resulted in >1.5 times the number of cells than with 0.1% collagenase. We had expected this difference because our previous results demonstrated that the efficiency of dedifferentiation into DFAT cells was higher for small adipocytes than for large adipocytes (23). Thus, a large number of DFAT cells can be obtained from the same number of small adipocytes compared with large adipocytes.

Finally, we compared MSC characteristics based on the number of CFU-Fs, immunophenotype, and differentiation capacity of cultures established using four different protocols based on different collagenase concentrations as viable DFAT cells were generated from mature adipocytes obtained from all protocols. Interestingly, ALP activity and calcium deposition analyses showed that the osteogenic differentiation potential of DFAT cells obtained using 0.02% collagenase was significantly higher than that of cells obtained using 0.01% collagenase, possibly because the proportion of small adipocytes digested with 0.02% collagenase was significantly higher than that digested using 0.01% collagenase in this study. In addition, our previous study demonstrated that DFAT cells generated from small adipocytes showed higher osteogenic potential than that of cells generated from large adipocytes. Surprisingly, we found that DFAT cells generated with 0.02% and 0.1% collagenase exhibited similar cell proliferation characteristics, gene expression patterns, colony forming efficiency, cell cycle patterns, and adipogenic differentiation capacities. Furthermore, we used tissues from 10 patients, but the age of the patients did not significantly influence cellular yield or viability (data not shown). The results suggested that DFAT cells generated using 0.02% collagenase are useful for bone or periodontal tissue engineering.

Our study focused on isolating adipocytes and DFAT cells from adipose tissue obtained from human BFPs; however, other studies have used subcutaneous tissue to isolate mature adipocytes for obtaining ASCs and DFAT cells. Further studies are therefore needed to determine the influence of collagenase concentration on the isolation of mature adipocytes from subcutaneous tissue.

In conclusion, we demonstrated the influence of collagenase concentration on the number of mature adipocytes isolated from BFP, and that 0.02% collagenase is optimal for this isolation. In addition, more mature adipocytes were obtained with 0.02% collagenase than with other collagenase concentrations. Furthermore, DFAT cells generated using 0.02% collagenase have a higher osteogenic potential.

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Conflict of interest

None declared.

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